High Performance Liquid Chromatography on Calixarene-Bonded Silica Gels. III. Separations of *cis/trans* Isomers of Proline-Containing Peptides

Sabine Gebauer[1], Sieglinde Friebe[1], Gerd Scherer[2], Gerald Gübitz[3] and Gerd-Joachim Krauss[1,*]

[1]Martin-Luther-University Halle, Department of Biochemistry/Biotechnology, Kurt-Mothes-Str. 3, 06120 Halle/S., Germany; [2]University of Basel, Institute of Organic Chemistry, St. Johanns-Ring 19, 4056 Basel, Switzerland; and [3]Karl-Franzens-University of Graz, Institute of Pharmaceutical Chemistry, Universitätsplatz 1, A-8010 Graz, Austria

Abstract

The liquid chromatographic behavior of *p-tert*-butylcalix[*n*]arene (*n* = 4, 5, 6, and 8) for the separation of *cis/trans* peptide bond isomers of proline-containing peptides is studied to demonstrate the chromatographic selectivity of calixarene stationary phases. The results are compared with the elution patterns obtained on RP18 and β-cyclodextrin, as well as on a monomeric *p-tert*-butylphenoxyacetic acid. The chromatographic data are established by rechromatography and ^{1}H-NMR spectroscopy. The results support the assumption that inclusion complexation seems to be a possible separation principle because of the size of the calixarene cavities.

Introduction

In recent years, calixarenes have gained increasing interest with cyclodextrins (CDs) and crown ethers in host-guest chemistry because of their extraordinary potential for the molecular recognition of neutral and ionic molecules. In previous papers, we pointed out several applications of calixarenes in HPLC (1,2). We introduced new calixarene-bonded silica gels for HPLC, demonstrating the resolution power and chromatographic selectivities based on application examples. Separations of regio- and stereoisomers depend on the shape and size of cavities supported by hydrophobic and π–π interactions.

The following investigations continue our work on the development of applications of the new calixarene phases for the separation of model substances such as proline-containing dipeptides (Ala-Pro, Phe-Pro, and Ile-Pro) in order to study the influence of calixarene ring size and its monomer structure. They extend former studies of the *cis/trans* isomerism of such peptides on β-CD (3–5) and on calix[4]arene stationary phases (1). Using CDs, the separation of *cis/trans* isomers of proline-containing peptides was decisively improved by the formation of inclusion complexes. This led to a complete discrimination of single isomers and ultimately to a removal of the plateaus between the peaks resulting from the transition state. To verify the separation mechanisms on calixarene phases, we decided to use these peptide studies. A comparison of elution profiles and some chromatographic parameters allows the estimation of separation principles on calixarene phases and the pointing out of their selectivity.

Investigations of proline-containing peptide conformers are of importance in understanding the regulation mechanisms of biological systems, including protein folding/refolding, immune responses, and opioid receptor recognition (6,7). Xaa-Pro peptide bond conformers can exist in two stable states, *cis* ($\omega \approx 0°C$) and *trans* ($\omega \approx 180°C$), because of their relatively high energy barriers of rotation around the peptidyl proline bond, which is attributed to the pyrrolidine ring. The interconversion rate of the conformers strongly depends on the amino acid residue bound to proline, the pH range, and the solvent composition. Other than NMR spectroscopy (8), CZE (9), and kinetic methods (6), HPLC is a helpful tool in studying the *cis/trans* isomerism of peptide conformers. The relaxation time of conformational change is slow enough and agrees with the time scale of the chromatographic run. Separation of the isomers can be optimized by varying the composition of the mobile phase in regard to buffer, organic modifier content (acetonitrile or methanol), pH value, and temperature.

Preliminary studies show that calixarene-bonded phases were able to resolve *cis/trans* isomers of peptides originating from the dynamic bond isomerization process (10,11). Separations of the *cis/trans* isomers of proline-containing oligopeptides by HPLC have been studied with many different chemically bonded stationary phases, such as several RP18 phases (12,13), β-CD (3,4), graphitized carbon, and L-proline Cu(II) phases (5).

In this paper, we demonstrate the chromatographic behavior of zwitterionic *cis/trans* dipeptides on *p-tert*-butylcalix[*n*]arenes (*n* = 4, 5, 6, and 8) bonded onto silica at 5°C. The results are compared with those obtained on RP18 and β-CD phases, as well as with a monomeric phase consisting of *p-tert*-butylphenoxyacetic acid, to elucidate the underlying separation principle. Rechromatography and ^{1}H-NMR spectroscopy were performed to estimate the existence and elution order of isomers on calixarene and β-CD stationary phases.

*Author to whom correspondence should be addressed.

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Experimental

Materials

Chemicals

Optically pure dipeptides (H-Xaa-Pro-OH {Xaa = Ala, Phe, Ile}) were purchased from Bachem Biochemica (Heidelberg, Germany). NaH_2PO_4, $NH_4H_2PO_4$, NaOH, NH_4OH, HPLC-grade acetonitrile, acetonitrile-d_3, and deuterated water were obtained from Merck (Darmstadt, Germany).

Columns

The calix[*n*]arene-bonded columns (250 × 4.6-mm i.d.) were prepared as previously described (2). Some characteristics of these stationary phases are listed in Table I. The column materials were stable in a pH range of 2 to 7, established by longtime experiments at these pH values and by a Tanaka method (14) using 30% buffered methanol at pH 2.7 and 7.6 for the test solutes thiourea, benzylamine, and phenol.

A β-CD=Si 100 (250 × 4-mm i.d., 5-μm particle size, 100-Å pore width) and an octadecyl=Si 100 (250 × 4-mm i.d., 5-μm particle size, 100-Å pore width) from Serva (Heidelberg, Germany) were used to compare the results of calixarene-bonded stationary phases. Preparative separations of Phe-Pro were also carried out on the β-CD=Si 100 phase (250 × 4-mm i.d., 5-μm particle size, 100-Å pore width) by using highly concentrated samples.

Table I. Characteristics of Calixarene-Bonded Stationary Phases

Parameter	[1]Arene*	[4]Arene†	[5]Arene†	[6]Arene†	[8]Arene†
Particle size (μm)	5	5	5	5	5
Mean pore width (Å)	100	100	100	100	100
Silica	irregular	irregular	irregular	irregular	irregular
Surface coverage (mmol/g)	0.51	0.15	0.10	0.14	0.09
pH stability	2–7	2–7	2–7	2–7	2–7

* Selector: Bu^t-phenoxyacetic acid.
† Selector: Bu^t-calixarene acetic acid ester.

Instrumentation

HPLC Apparatus

Chromatographic separations were performed on a Merck-Hitachi HPLC system consisting of an L-6200 intelligent pump, a 655 variable wavelength ultraviolet detector, and data acquisition software (Merck-Hitachi Model D-6000, HPLC-Manager Version 2). A Julabo VC constant temperature bath (Seelbach, Germany) regulated the cooling of the columns at 5°C.

NMR

^1H-NMR experiments were carried out on a Bruker ARX 500 NMR spectrometer with proton resonance frequency at 500.13 MHz. All measurements were performed at 4°C. For temperature calibration, a Keithley 866 thermometer embedded in an NMR tube was used. NMR spectra were recorded in the Fourier transform mode (16,000 data points and 256 scans). Samples contained 0.03–0.2 mg per 600 mL of peptides dissolved in a D_2O–acetonitrile-d_3 mixture (95:5, v/v).

Chromatographic procedures

Analytical HPLC

Chromatographic experiments were performed isocratically using 0.02M sodium or ammonium dihydrogen phosphate buffer–acetonitrile eluents of different compositions. In all cases, the pH value was adjusted to 6.2 with NaOH or NH_4OH. The samples were dissolved in the eluents at a concentration of 0.25 mg/mL. Prior to measurement, the samples were equilibrated with the mobile phase at room temperature for 1 h and at the column temperature for 30 min. The absorption of analytes was monitored at 210 nm.

The influence of the organic modifier content of mobile phase was investigated in the range of 0 to 7% acetonitrile. The column temperature was varied from room temperature down to 5°C. The injection volume was 20 μL and the flow rate was set at 1 mL/min. All measurements were repeated

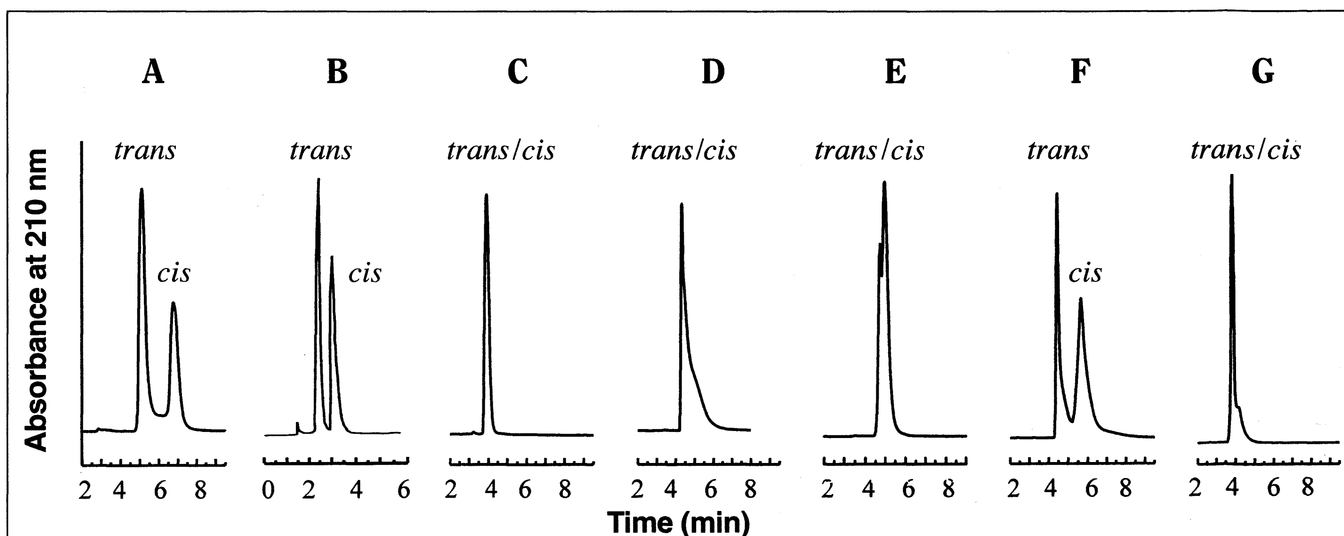

Figure 1. Isocratic separations of Ala-Pro at 5°C on different stationary phases using 0.02M $NH_4H_2PO_4$ (pH 6.2) at a flow rate of 1 mL/min and an absorbance at 210 nm. Octadecyl=Si 100 (A), β-CD=Si 100 (B), [1]Arene Si 100 (C), [4]Arene Si 100 (D), [5]Arene Si 100 (E), [6]Arene Si 100 (F), [8]Arene Si 100 (G).

at least twice, then once again after a certain amount of elapsed time to exclude defects of column materials.

Preparative HPLC

Preparative HPLC was carried out to re-chromatograph the pure isomers on calix[8]arene-bonded phases to show that peak splitting is a result of isomerism. The mobile phase was 0.02M $NH_4H_2PO_4$ buffer containing 5% acetonitrile. The flow rate was 1 mL/min. Furthermore, preparative chromatography was used to demonstrate the elution order on a β-CD phase by means of ^{1}H-NMR spectroscopy. The mobile phase consisted of D_2O–acetonitrile (95:5) and the flow rate was 0.8 mL/min. Samples contained 1 mg/100 µL peptide to fractionate the first eluting peak and 0.1 mg/100 µL peptide to fractionate the second. Fractions of 600 µL isolated isomers were collected in NMR tubes at 0°C and measured immediately by ^{1}H-NMR spectroscopy at 4°C.

Results and Discussion

HPLC of Ala-Pro

It is known that the isomeric equilibrium and the rate of isomerism are strongly influenced by organic modifier content, temperature, and pH value. With increasing organic modifier content and temperature and decreasing pH value, the interconversion of isomers is accelerated (15). Furthermore, the *cis/trans* isomer ratio is changed remarkably at acidic pH. Therefore, for the separation of the small hydrophilic isomers of Ala-Pro, the modifier content was varied only from 0 to 5% acetonitrile at pH 6.2. Best results were achieved without any organic modifier content of the mobile phase. Figure 1 shows the chromatographic separations on different stationary phases (RP18, β-CD, [1]-, [4]-, [5]-, [6]-, and [8]Arene; the notation [*n*]Arene was chosen for the monomeric and calixarene-bonded materials, and the *n* in brackets represents the number of aromatic units in the calixarenes). Compared with RP18 phases, baseline separations were also achieved on β-CD and [6]Arene. A peak splitting was observed using [5]Arene. We assume that the separation and the peak splitting are a result of the discrimination of isomers by the chromatographic support (at room temperature, the isomers were co-eluted as a single peak because of the rapid interconversion). These selectors influence the distribution equilibrium by steric discrimination. The comparable elution patterns on β-CD and [6]Arene are possible because of the same ring size (possibly an indication of inclusion complex formation). No selectivity is observed on [1]-, [4]-, and [8]Arene. Only peak shoulders indicate the existence of peptide isomers. The selectors probably do not have the right dimensions and necessary properties to discriminate between the isomers. It has to be assumed that the cavity of [4]Arene is too small for an intermolecular interaction with Ala-Pro just as [5]Arene. On the other hand, the calix[8]arene might be too large and flexible (16), so a selective contact is not possible.

On the RP18 phase, the peaks were well separated, but the baseline did not get the ground line. That means that the isomerization interferes with the chromatographic process. The plateau represents the transition state. This phenomenon, shown with porous RP18 material, indicates mass transfer problems. It may be overcome by using stationary phases which separate via inclusion complexes, such as β-CD and [6]Arene.

HPLC of Phe-Pro

The behavior of Phe-Pro on stationary phases is shown in Figure 2. The organic modifier content of mobile phase was varied from 0 to 7% acetonitrile. The use of pure phosphate buffer as mobile phase reduces the selectivity. The mobile phase containing 7% acetonitrile produces a sufficient baseline separation in a relatively short time on the calixarene-bonded silica gel, with a resolution of 2.8 achieved on [8]Arene. The isomers are clearly separated in all cases. Because of the hydrophobic and bulky side chain of Phe-Pro, the rotational barrier is relatively high and agrees well with the chromatographic time scale. Demands on the stationary phases are not very high.

With increasing numbers of aromatic moieties and *tert*-butyl

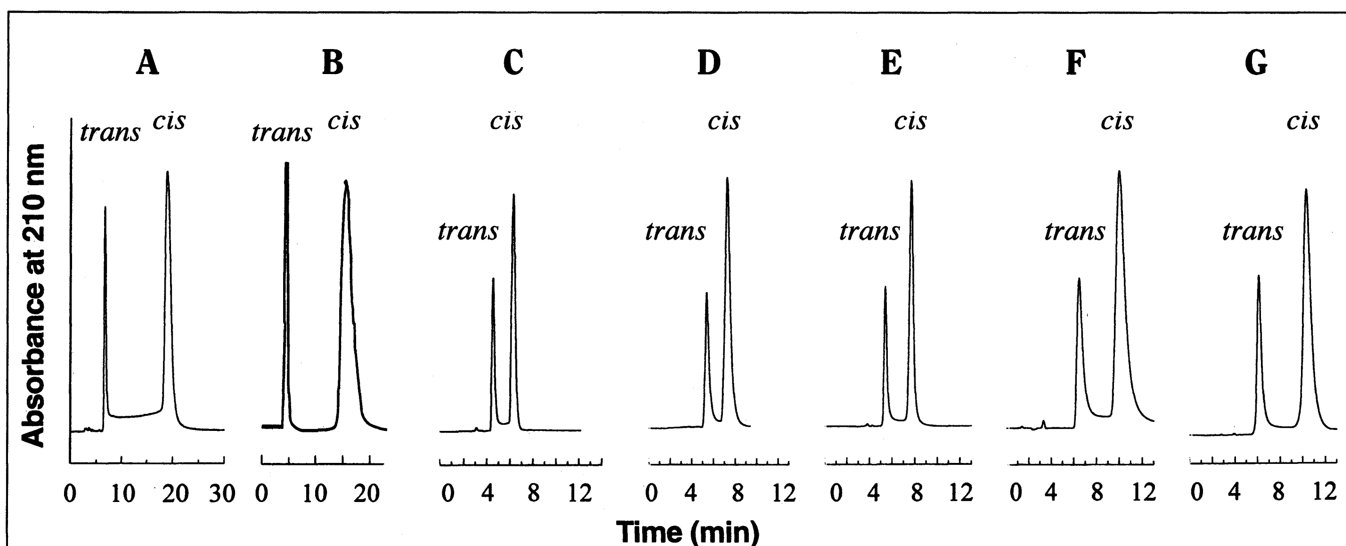

Figure 2. Isocratic separations of Phe-Pro at 5°C on different stationary phases using 0.02M $NH_4H_2PO_4$ (pH 6.2)–acetonitrile (93:7, v/v) as a mobile phase at a flow rate of 1 mL/min. The isomers were monitored at 210 nm. Octadecyl=Si 100 (A), β-CD=Si 100 (B), [1]Arene Si 100 (C), [4]Arene Si 100 (D), [5]Arene Si 100 (E), [6]Arene Si 100 (F), [8]Arene Si 100 (G).

groups of the calixarenes, the resolution is improved because of a favored "hydrophobic interaction potential". The peak ratios are comparable in all cases and were found to be about 25:75 (± 3) area percent. The observed results may be attributed to an interaction between the hydrophobic side chain of the dipeptide and the *tert*-butyl groups of the smaller calix[*n*]arenes (n = 4 or 5) or the hydrophobic cavities of larger calix[*n*]arenes (n = 6 or 8), respectively. However, formation of host-guest complexes on the [6]- and [8]Arene cannot be excluded.

Under the same conditions, the isomers of Phe-Pro are retained very strongly on the RP18 phase due to the hydrophobic forces. The elution pattern shows a plateau (note the rising baseline) between the peaks as a consequence of an insufficient discrimination of the isomers (compared with the separation of Ala-Pro). The plateau also represents the transition states between *cis* and *trans* (17). The effect is more significant because of the long elution time of the *cis* isomer. Using higher contents of organic modifier, the peaks were eluted earlier and the plateau was smaller, but the isomeric ratio was changed dramatically (15,18). An improvement of this result comparable to that of β-CD resolution was possible by optimizing the silica gel material (e.g., a non-porous, micropellicular RP18 silica gel) (13).

HPLC of Ile-Pro

The chromatographic behavior of the dipeptide Ile-Pro on calixarene-bonded phases was also demonstrated (Figure 3). The mobile phase composition was optimized in a range from 0 to 7% acetonitrile. Retention decreased with increasing modifier content. Best resolutions were achieved with 5% acetonitrile on [5]- and [8]Arene with a resolution of approximately 1. The results display a sufficient retention but no baseline separations. The peaks were very sharp and symmetrical. Interestingly, the ratio of *cis* and *trans* peaks were not the same in all cases. This may be attributed to the influence of a second dynamic process, such as the different chromatographic distribution of the isomers between the stationary and mobile phase.

The investigation of the porous RP18 phase shows the typical plateau between the isomeric peaks which was also observed with the Phe-Pro separation.

Influence of buffer composition of the mobile phase

Since calixarene esters are known to show a distinct selectivity for different cations (19), it was of interest to investigate the influence of the buffer cation on the selectivity of the calixarene-bonded silica gels. Calix[4]arene esters display a high selectivity for Na^+ ions, whereas calix[6]arene esters show a better complexation of Cs^+. For this reason, NaH_2PO_4 and $NH_4H_2PO_4$ solutions were applied as mobile phases containing different amounts of acetonitrile. Indeed, the chromatographic selectivity (a) of calixarene phases was improved by choosing the suitable cation for each calixarene. A higher selectivity for Na^+ ions was observed on [4]Arene (Figure 4). All measurements using a NaH_2PO_4 solution as a mobile phase showed a stronger retention and a better resolution of the dipeptides. Therefore, it is very useful to carry out chromatographic investigations on the [4]Arene phase with sodium-containing mobile phases; sodium complexation results in a better preorganization of the basket-like shape because of the complexation of sodium by phenolic and carbonyl oxygens of calix[4]arene tetraester immobilized in cone conformation. Thus, the C_{2v} symmetry of the cone conformation is changed to C_{4v} (19).

A similar effect was observed for [6]Arene with $NH_4H_2PO_4$ solution as a mobile phase. However, the selectivity is generally less pronounced than that achieved with [4]Arene and sodium (Figure 4) at 2% acetonitrile.

Selective complexation properties of calix[8]arene esters are not described in the literature, but chromatographic separations were improved with an $NH_4H_2PO_4$ solution as mobile phase using higher acetonitrile contents. This is seen in Figure 4 at all acetonitrile concentrations ranging from 2 to 7%. Generally, higher a values were obtained.

Furthermore, the influence of water–acetonitrile mixtures as mobile phases for the separation of Phe-Pro on [8]Arene was investigated by means of the separation of Phe-Pro as a model

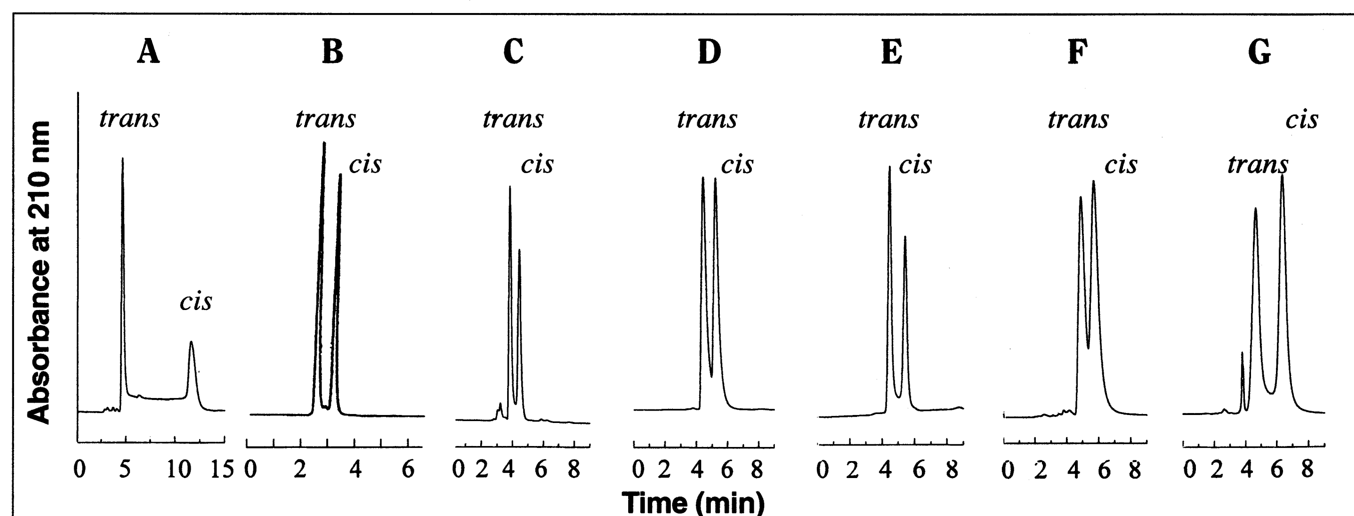

Figure 3. Isocratic separations of Ile-Pro at 5°C on different stationary phases using 0.02M $NH_4H_2PO_4$ (pH 6.2)–acetonitrile (95:5, v/v) as a mobile phase at a flow rate of 1 mL/min. The detection wavelength was 210 nm. Octadecyl=Si 100 (A), β-CD=Si 100 (B), [1]Arene Si 100 (C), [4]Arene Si 100 (D), [5]Arene Si 100 (E), [6]Arene Si 100 (F), [8]Arene Si 100 (G).

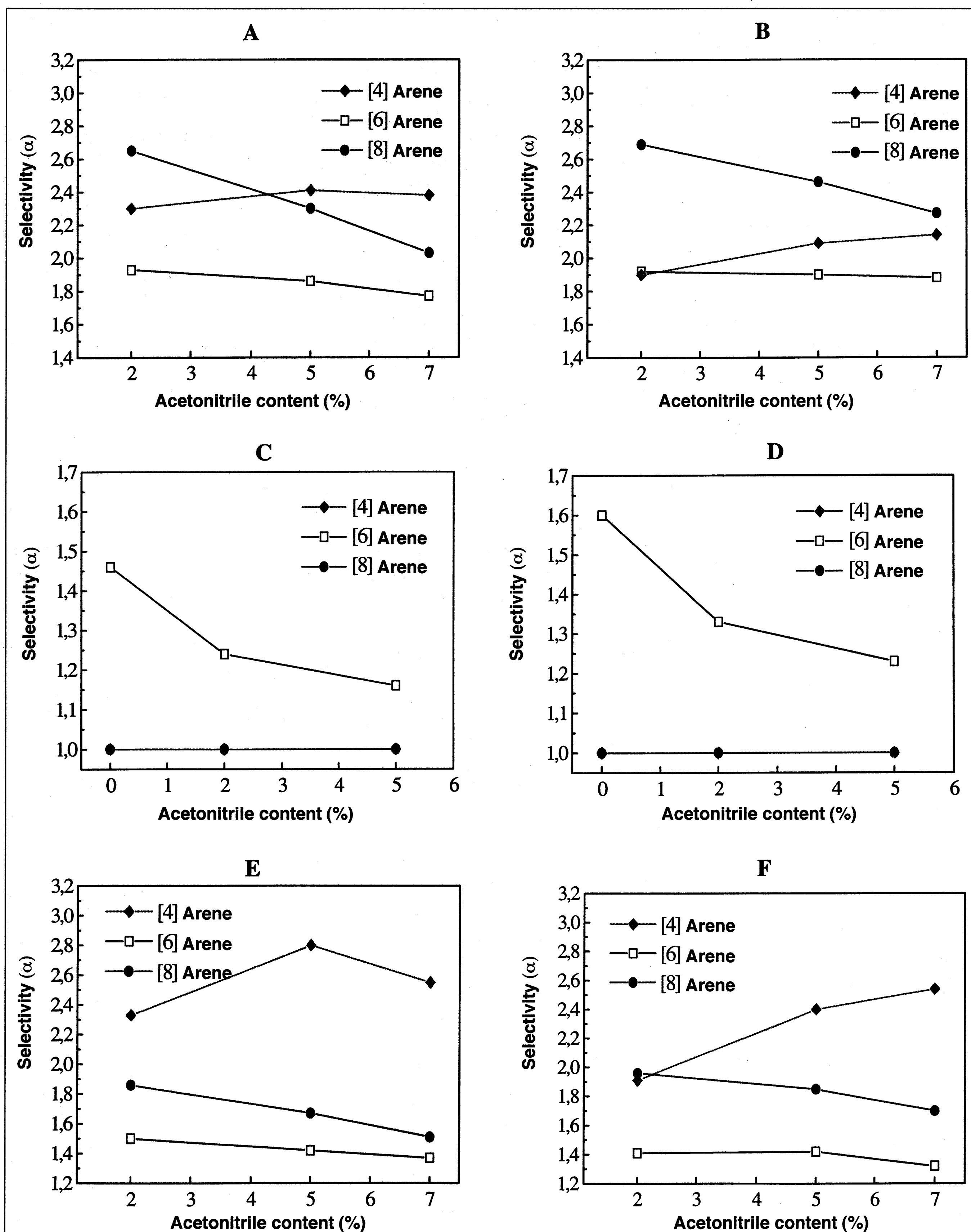

Figure 4. Effects of the mobile phase composition on *cis/trans* isomer separations. Comparison of selectivity (α) of dipeptides versus acetonitrile content for Phe-Pro (A,B), Ala-Pro (C,D), and Ile-Pro (E,F). The mobile phases were 0.02M NaH_2PO_4 (A,C,E) and 0.02M $NH_4H_2PO_4$ (B,D,F).

peptide. However, the peaks were less sharp and more rapidly eluted. This indicates that buffer systems as mobile phases are necessary not only for ion pair formation of the zwitterionic dipeptides with the mobile phase, but also for cation complexation (Na^+ or NH_4^+ ions) by the ester ligands of the selector, which resulted in a better preorganization of this receptor molecule.

Identification of isomeric peaks by rechromatography and ^{1}H-NMR spectroscopy

Rechromatography

In order to confirm that the peaks are *cis* and *trans* isomers, rechromatography on [8]Arene was done with Phe-Pro. This stationary phase was chosen because of its high resolution. The latter eluting main fraction was re-injected. The resulting chromatogram showed a smaller amount of the first peak (7.5 area percent) than the parent (25 area percent). After storage of the fractionated isomer peak at room temperature for one hour and cooling down to 5°C for 30 min, re-injection of this fraction showed that the reversible interconversion equilibrium of *cis* and *trans* isomers was set to give the same proportion as in the original sample (25:75 area percent).

^{1}H-NMR spectroscopy

To identify isolated *cis/trans* isomers by HPLC, low temperature ^{1}H-NMR spectroscopy studies were carried out. The *cis/trans* isomerism of Phe-Pro is slow enough to distinguish between the *cis* and *trans* conformers.

As mentioned above, the fractionation of isomers using water–acetonitrile as mobile phase on [8]Arene was very difficult because the resolution decreased. We decided to overcome this disadvantage by using a β-CD phase. For preparative separation with deuterated eluents, this stationary phase was overloaded with 1 and 0.1 mg Phe-Pro dissolved in 100 µL mobile phase, respectively. The isolated fractions were measured immediately by NMR spectroscopy at 4°C. The ^{1}H-NMR signals of Phe-Pro for the first fraction can be assigned to the *trans* isomer and the second fraction to the *cis* isomer (Figure 5), which agrees with published results by Kálmán et al. (13). Furthermore, the obtained ^{1}H-NMR data confirm the assumption of our former investigations on β-CD that the *cis* isomer is more strongly retained than the *trans* (3). The elution order of conformers on a calixarene-bonded silica was determined by the immediate re-injection of the main fraction onto the [8]Arene. The same elution order as observed on β-CD was estimated.

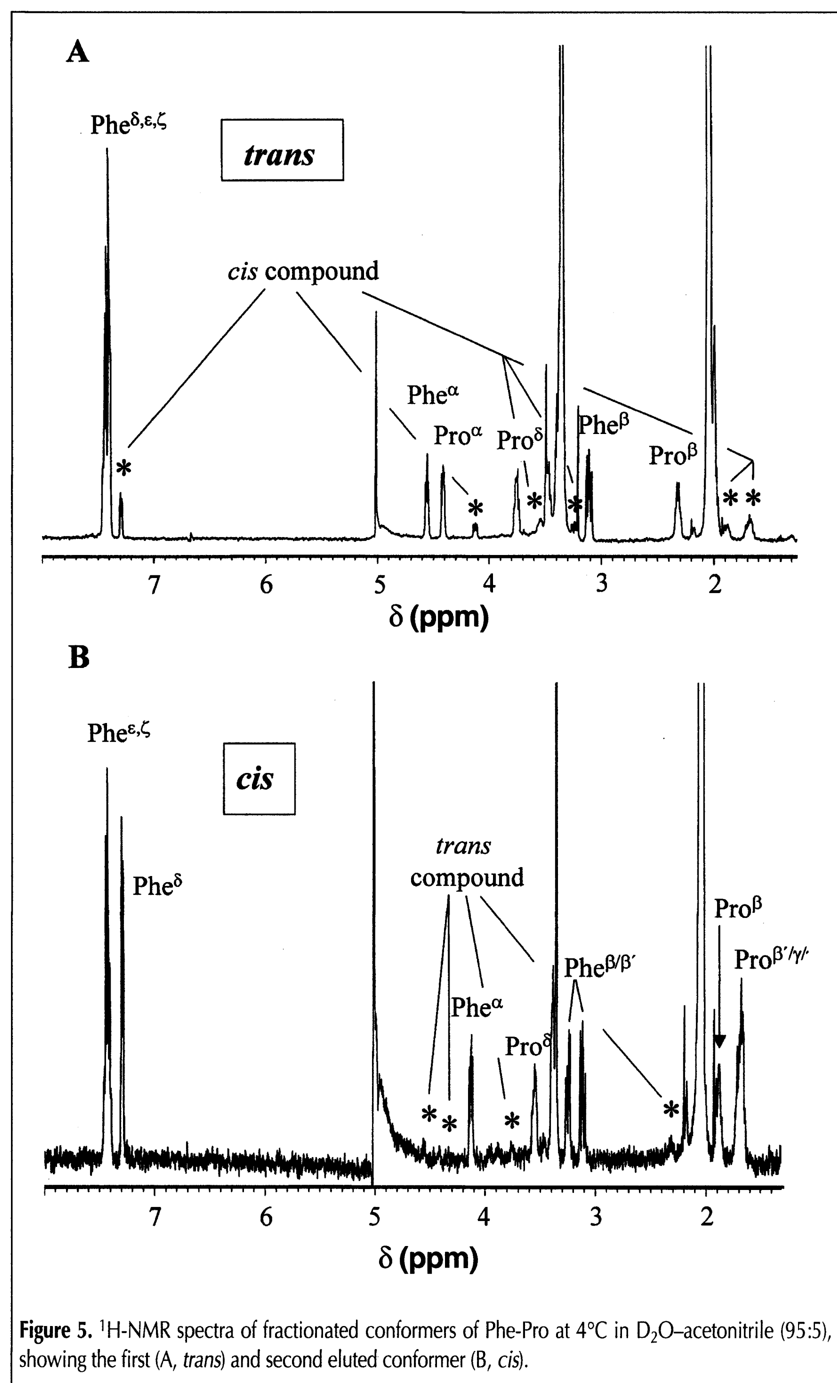

Figure 5. ^{1}H-NMR spectra of fractionated conformers of Phe-Pro at 4°C in D_2O–acetonitrile (95:5), showing the first (A, *trans*) and second eluted conformer (B, *cis*).

Conclusion

Low temperature chromatographic separations of proline-containing dipeptides (Ala-, Phe-, and Ile-Pro) were performed on calix[*n*]arene (n = 4, 5, 6, 8) stationary phases. The results show similarities to highly specific β-CD and RP18 phases. Calixarene-bonded silica gels display a reversed-phase behavior first and foremost. Furthermore, these results indicate a high selectivity of the calix[*n*]arene phases dependent on the ring size. The differences in elution patterns in comparison with reversed-phase chromatography may be a result of a host–guest complexation. For instance, Ala-Pro was only separated by the use of the [6]Arene column, whereas resolution of dipeptides with bulky, hydrophobic side chains was increased on the [8]Arene phase.

The composition of mobile phase (cation and acetonitrile content) has shown a pronounced

influence on the resolution of the dipeptide conformers.

Rechromatography confirmed that the peaks were caused by *cis/trans* isomerism of the peptides. The elution order of *cis* and *trans* conformers of Phe-Pro could be established by ^{1}H-NMR spectroscopy at lower temperatures. HPLC investigations of proline-containing dipeptides with the calixarene-bonded silica gels demonstrate that these materials are useful tools for the development of separation methods for *cis/trans* conformers.

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